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MADAM Protein Decreases Microsporidia Attachment to Host Cells

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ABSTRACT

Microsporidia are an obligate, intracellular fungal pathogen that can cause devastating, disseminating infections in the immunocompromised. Because of the limitations of current medications, microsporidia's abundant presence in the environment, and an increasing number of at-risk populations, investigation into decreasing microsporidia infectivity is needed. As an intracellular pathogen, microsporidial attachment is a vital first step to infection, and if attachment is reduced, previous work shows that infectivity is mitigated. An *in silico* analysis of *Encephalitozoon intestinalis* revealed a predicted protein similar in sequence to ADAM (A Disintegrin And Metalloproteinase) proteins. This predicted protein is termed microsporidia ADAM or MADAM. ADAM proteins contain an integrin binding region, which is well known to bind to integrin proteins. Integrins are important receptors for attachment and cell signaling, and several pathogens utilize host integrins as a receptor to aid in attachment during infection. Immunoelectron microscopy demonstrates that MADAM protein is found on the plasma membrane, anchoring disk, and polar tube of *E. intestinalis* spores. Our hypothesis is that MADAM is involved in the key role of host cell attachment. To this end, a 17 amino acid long section of the MADAM protein was generated that surrounded the integrin binding domain. During spore adherence assays, pretreating host cells with this small peptide protein, significantly decreased *E. intestinalis* spore attachment to host cells as compared to control samples. These results suggest *E. intestinalis* cleverly exploits host integrins as a means to bind to host cells before infection.

INTRODUCTION

Microsporidia have an extensive range with over 1,400 species, and they are responsible for severe clinical consequences in high-risk populations.^{1,2} The key virulence factor for microsporidia is its highly resistant spore that allows the pathogen to remain viable and infectious for several years outside of its host.³ A unique trait common to all microsporidia is the polar tube, a mechanism through which infectious sporoplasm is released from the spore into host cells.⁴

In humans, the symptoms of microsporidial infection can be easily overlooked as self-limiting traveler's diarrhea.⁵ However, a more serious infection occurs in the immunocompromised, where patients often fail to clear the infection and a chronic disease state with hallmarks of persistent diarrhea and wasting occur, sometimes in spite of antifungal treatment.^{6,7} The most serious clinical presentation of uncontrolled, lethal dissemination also occurs in the immunocompromised.⁸ Unfortunately, there are a limited number of treatments available once an infection is acquired.⁹ Moreover, current antifungal therapies like Albendazole are not effective against all clinically relevant microsporidia,⁸ and stronger medications, such as Fumagillin, have toxic side effects.³ Although it is an obscure pathogen in some areas, prevalence of microsporidia remains high in countries that do not have widespread distribution of antiviral therapy to HIV (human immunodeficiency virus) patients.^{5,10} This pathogen will likely gain more attention in the future, as immunosuppression has become a prescribed therapy for some chronic diseases and researchers expect the immunosuppressed population to increase.¹¹

Previous work shows microsporidia utilize several mechanisms to attach to host cells, as spore proteins can bind to glycosaminoglycans, mannose, and transferrin receptors.^{12,13,14} Given the limited number of therapeutics, more research into microsporidial attachment is necessary, as previous work demonstrates if attachment of microsporidia is reduced, then infection is decreased.¹⁵

This study seeks to elucidate a novel mechanism of attachment used by microsporidia spores during host infection. Upon *in silico* investigation for attachment motifs, the MADAM protein was identified. Subsequent immunoelectron microscopy experiments demonstrate MADAM is expressed on the spore anchoring disk and polar tube, making the protein host exposed (unpublished). MADAM is similar in sequence to other ADAM proteins, as both contain an integrin binding domain.¹⁶ ADAMs are a diverse group of proteins with several functions including proteases, cell binding, and signaling.¹⁶ Their binding target—integrins—are also involved in host cell signaling and attachment; yet, many bacterial and viral pathogens exploit integrins for their attachment to host cells.^{17,18}

Our hypothesis is that microsporidia also utilize integrins as a means to attach to host cells during infection. To test this, the 17 amino acid sequence surrounding the integrin binding domain of the MADAM protein was generated and used in spore adherence assays. Because of the limitations of current medications, insights gained during primary research into spore attachment can offer tools for molecular research and targets for future therapeutic development.

MATERIALS & METHODS

- Peptides**
- A MADAM peptide was generated by selecting the 17 amino acid sequence that surrounded the integrin binding domain of the complete MADAM protein: [NH₂] RGRSTNIHKMDCERESY [COOH]. Reference number for the entire MADAM protein: ECU08_3080.
 - A Control peptide was generated by selecting 17 amino acids at random: [NH₂] ETGCMRSKYEDRIHNSR [COOH]. Both peptides were generated by Thermo Scientific.
- Predicted structures of the MADAM and Control peptides** were created in Discovery Studio Visualizer software (Figure 1A and 1B).
- Experimental Setup of Spore Adherence Assays**
- Vero cells (ATCC) were grown to confluence on glass coverslips in 12 well plates. Cells were either pretreated with MADAM or Control peptides for one hour at 37°C with decreasing concentrations of peptides (10 to 0.000001 µg peptides/mL). A control group was not exposed to any peptide, instead 10 µL of PBS was used. After pretreatment, *E. intestinalis* spores (ATCC) were added at 1 x 10⁷ spores/well, and the wells were placed on ice. The spores were allowed to adhere to the host cells for four hours. Afterwards, each coverslip was washed in PBS for 30 seconds, fixed with a 50:50 ratio of Acetone: Methanol, and allowed to dry. The spores and cells were visualized by Uvitex 2B and DAPI staining.
- Data Analysis**
- Spores within ten reticles were counted per coverslip, per experiment at 630X oil immersion. Three experiments were performed. The data of each experiment was combined and analyzed by ANOVA and Tukey post hoc testing. A *p* value of <0.05 was used to determine statistical significance. SPSS software was used for analysis and to generate graphs.
 - Non-parametric Kruskal-Wallis with Dunn-Bonferroni post hoc testing was completed (not shown); however, ANOVA testing is reported here for ease of interpretation, as both parametric and non-parametric tests yielded similar results.

FIGURE 1

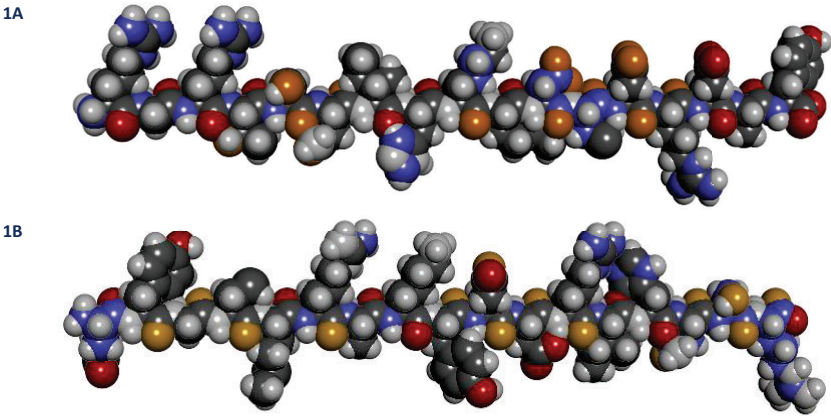


Figure 1. Predicted structures of the MADAM (A) and Control peptide (B) used in spore adherence assays. Models created using Discovery Studio Visualizer.

FIGURE 2

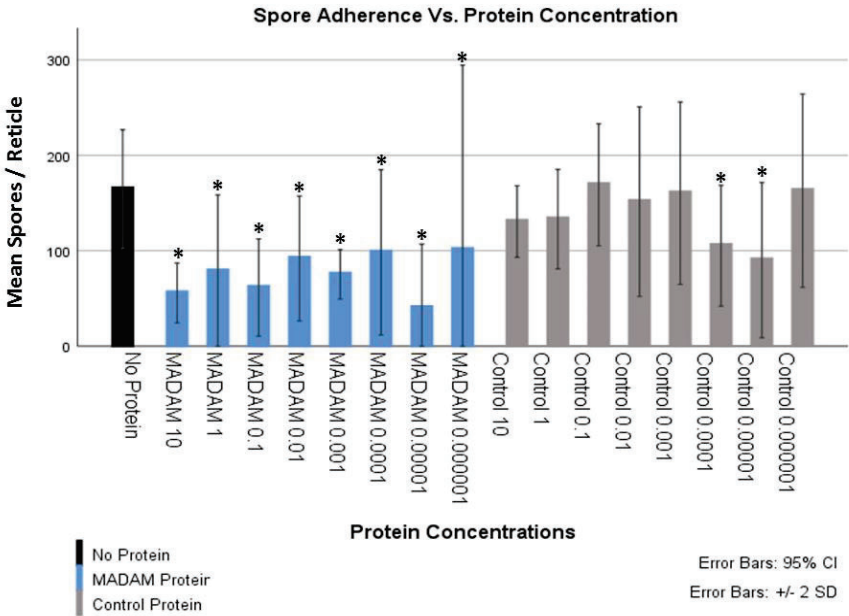


Figure 2. Histogram of mean spore adherence of *E. intestinalis* spores to Vero cells that were exposed to decreasing levels of MADAM or Control peptides. A positive control group was not exposed to any peptide. The number of spores within ten reticles per coverslip, per experiment were counted. Data from three experiments was compiled and analyzed by ANOVA and Tukey post hoc testing. Asterisks indicate cells that have significantly lower spore adherence compared to the untreated “No Protein” control. CI = confidence interval; SD = standard deviation.

RESULTS

A difference in spore adherence was seen between cells exposed to different peptides (MADAM, Control, or no peptide) determined by ANOVA testing (F (16,315) = 18.947, **p < 0.000**).

MADAM Peptide Vs. Untreated Cells (Figure 2)

- Pretreatment with MADAM peptide significantly lowered average spore adherence per reticle from **164.6 spores** on untreated cells to **55.8 spores** on cells pretreated with MADAM 10 µg/mL (Tukey post hoc testing ± 15.7 spores, **p < 0.000**).
- Indeed, pretreatment with **all concentrations** of MADAM peptide significantly lowered spore adherence compared to untreated cells, **p < 0.000** for all concentrations.
- With one exception (MADAM 0.00001 µg/mL), there was no statistically significant difference in spore adherence among MADAM protein concentrations (p = 0.21100 to 1.00).
- The exception: spore adherence of cells pretreated with MADAM 0.00001 is statistically lower than cells pretreated with MADAM 0.01 (p = 0.012), MADAM 0.0001 (p = 0.002), and MADAM 0.000001 µg/mL (p = 0.001).

Control Peptide Vs. Untreated Cells (Figure 2)

- In general, there was no difference in spore adherence between untreated cells and those pretreated with the Control peptide (p = 0.29 to 1.00).
- The exceptions: spore adherence of cells pretreated with Control 0.0001 and Control 0.00001 µg/mL concentrations had lower spore adherence than the cells that were not exposed to peptides (p < 0.000).
- These same concentrations had lower spore adherence than the four other Control peptide concentrations: 0.1, 0.01, 0.001, and 0.000001 µg/mL (p < 0.000 to 0.05).

Caveats

- Replicates of these experiments should be conducted, as an increase in sample size may reduce the variance seen in some protein concentrations (specifically the MADAM 0.000001 µg/mL seen in Figure 2).
- It is also suspected that a larger sample size would show the spore adherence of cells exposed to **all** concentrations of the control peptide would not be statistically different than the control group, which was not exposed to any peptide (Figure 2, Control Groups 0.0001 and 0.00001 µg/mL).

DISCUSSION

These experiments offer additional insight into the mechanism used by microsporidia for their attachment to epithelial cells. The MADAM peptide used in this experiment was selected because of its predicted integrin binding domain and its host exposed localization on the spore anchoring disk and polar tube. Cells pretreated with MADAM peptide had significantly lowered spore adherence compared to untreated cells. Given the intrinsic integrin binding domain of the MADAM *protein* present on the spore, and the decreased spore adherence when cells are pretreated with the MADAM *peptide* (that contains the exact integrin binding domain), this would suggest that the peptide competitively binds a host cell integrin to decrease pathogen binding.

It is known that microsporidia attach to mannose, transferrin, and glycosaminoglycan receptors.^{12,13,14} The experiments outlined here suggest microsporidia also exploit integrin binding as a novel mechanism for attachment. It is advantageous that microsporidia utilize several different mechanisms to bind host cells because host cell binding is an essential first step for most intracellular pathogens.¹⁹

Although research of microsporidia has been ongoing for 150 years,²⁰ scientists still have many problems to resolve and much more to discover before the complete pathway of microsporidial attachment and host cell invasion is described.

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